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13. ABSTRACT (Maximum 200 words)

Investigations on the in situ biodegradation of nitroaromatic compoundc have progressed nicely. Laboratory studies have identified the primary intermediatre compounds from anaerobic metabolism to be hydroxyaromatic compounds that are all biodegradable. Studies have identified the environmental parameters affecting the initial transformation of TNT and RDX in anaerobic cultures. Optimum pH and temperature for biodegradation is a pH of 8 and temperatures of 25-35 degrees C. Microbiological studies will continue to determine ways of enhancing the biodegradation of these compounds.

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Status Report
In-situ Biodegradation of Nitroaromatic Compounds in Soil
Principal Investigators: R. Crawford, D. Crawford, R. Korus
Grant AFOSR-91-0315
July 14, 1992

Introduction

The research performed in our laboratories on the anaerobic remediation of munitions-contaminated soils is summarized below. Most of these results were presented at the 1992 American Society of Microbiology General Meeting in New Orleans (copies enclosed). The presentations were viewed with much enthusiasm at the meeting, and were among a total of seven posters concerning the bioremediation of munitions compounds. Ours were the only studies dealing with actual contaminated soils as opposed to studies using pure TNT in synthetic medium. We are currently writing two manuscripts for submission to peer-reviewed journals for publication.

We have received a new soil (from Weldon Springs, Missouri) from Capt. Charles Coyle of the Army Corps of Engineers for treatability studies. These studies are now under way. The soil has a large clay component and initial experiments have indicated that the desorption of the contaminants from this soil is much slower than from the Umatilla, Oregon, soil used in our previous experiments. It will be interesting to compare the results of this study to those from the sandy Umatilla soil, which allows quick desorption of the contaminants. This comparison will help in developing a model describing the effects of the physical desorption rates on the biological activities and total treatment times that can be used to design reactors and treatment procedures.

Summary of Research since Last Report

During the past year many of the TNT biotransformation intermediates found in the culture supernatants of our anaerobic cultures have been identified or tentatively identified. Figure 1 summarizes the intermediates we have detected in the aqueous phase of soil slurries during the course of treatment. The identification of non-nitrogen-containing hydroxyaromatic intermediates formed from TNT will be a major accomplishment. These hydroxyaromatic compounds are all biodegradable under both aerobic and anaerobic conditions. Complete identification of these compounds will lead to a better understanding of the concurrent metabolic activities occurring in the culture vessels.

We have also examined the environmental parameters affecting the initial transformations of TNT and RDX in anaerobic cultures inoculated with soil acclimated to the degradation of dinoseb. Experiments to optimize the initial stage of TNT degradation, i.e. the removal of TNT, 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene and RDX, have shown that these compounds are removed from soils more rapidly at temperatures from 25-35°C, and with 10-25 mM added ammonium as a supplemental nitrogen source. The initial stage of soil remediation is now regularly completed by approximately 15-25 d incubation when the incubation starts with approximately 100 mg of TNT per liter of buffer (Figure 2). Determinations of the optimal pH for the removal of munitions compounds from soil have shown that although the initial compounds and initial intermediates are removed rapidly at pH 8, at least 50% of the ^{14}C from ^{14}C-U-ring-labeled TNT can be recovered as a nonfilterable material, presumably polymerized or precipitated intermediates (Table 1). At pH 6 no nonfilterable material was detected, although metabolism of the intermediates was slower, requiring 50-60 d to complete the initial stage.

We have also been investigating the development and maintenance of munitions-degrading microbiological inocula. One such inoculum that shows promise is a strictly anaerobic aqueous culture. The culture is periodically fed an extract from a munitions-contaminated soil in a mineral feed solution. The only source of carbon is the munition extract. This inoculum is capable of rapidly removing TNT, RDX, and other munitions compounds in the soil extract feed solution (unpublished results). The cul-

ture is presently at a volume of 4 liters and is being scaled up by the addition of feed allotments containing an extract of munitions-contaminated soil and mineral medium. Metabolic intermediates other than the amino-nitro compounds usually observed have been produced, and are further along the catabolic pathway than the previously known intermediates. The compounds 2,4,6-trihydroxytoluene and *p*-cresol have been identified in the culture supernatant. There are still other unidentified compounds (Figure 1).

An anaerobic inoculum has also been obtained from a munitions-contaminated soil culture. The culture appears to contain one or more organisms that metabolize TNT along one or more pathways different from those of the usual initial reduction mechanisms observed (unpublished results). Aqueous enrichment cultures of sheep rumen fluid in medium containing an extract of munitions-contaminated soil have also been established. Dr. A. M. Craig of Oregon State University has observed that such cultures degrade TNT and may employ unique pathways. We have been collaborating with his group on this possible useful microbial system.

Microbiological techniques have been used to purify 15 isolates from the aqueous anaerobic enrichment culture. Investigations to determine the identities and functions of these isolates are currently under way. This will allow us to begin looking at the roles of individual bacterial strains in the complex TNT/RDX biodegradation process.

Several experiments are currently under way with the Weldon Springs soil, which contained no detectable RDX or HMX. Incubations are about 4 weeks old. In one, a blend of contaminated and uncontaminated soil (approximately 20,000 mg TNT/kg soil) was loaded at 5.0% (w/v) and incubated at 30°C and pH 7.0. Half of the flasks have been inoculated with our anaerobic, TNT-degrading aqueous culture. This experiment will allow us to determine whether we need to inoculate this soil to obtain and/or enhance TNT degradation rates. Sampling data through 4 weeks show that this soil can be bioremediated, and the inoculated and noninoculated cultures are acting similarly. No TNT remained in the cultures after 6 days of incubation. The primary intermediate still present after 6 days was 2,4-diamino-6-nitrotoluene. After 3 weeks, this intermediate was also gone. Thus, TNT degradation appears to be going to completion. Half of these flasks have now been converted to aerobic conditions, to investigate the effectiveness of aerobic vs. anaerobic conditions in the second stage of soil remediation.

Future Research Directions

We plan to concentrate on optimizing the second stage (removal of the triaminotoluene and hydroxyaromatic intermediates) of the remediation of contaminated soils. We may institute a second stage (aerobic or nitrate-reducing) to allow a more rapid removal of these compounds. The exact time during the incubation to introduce the second stage and the levels of oxygen or nitrate required will be examined. We are also setting up some enrichment cultures on the target intermediates to ensure that a capable inoculum is available.

We also plan to continue the treatability study with the Weldon Springs soil. Planned experiments include investigations using larger quantities of soil with higher TNT concentrations in the soil. We will also continue to investigate what conditions are necessary to enhance the second stage of soil remediation.

Microbiological examinations will continue. We will identify isolated strains to at least the genus level, and examine the ability of each pure culture to transform TNT, RDX, HMX, 2,4-diamino-6-nitrotoluene, and *p*-cresol. These experiments will allow us to determine if our pure cultures are primary effectors of munitions degradation in the original consortium.

Figure 1

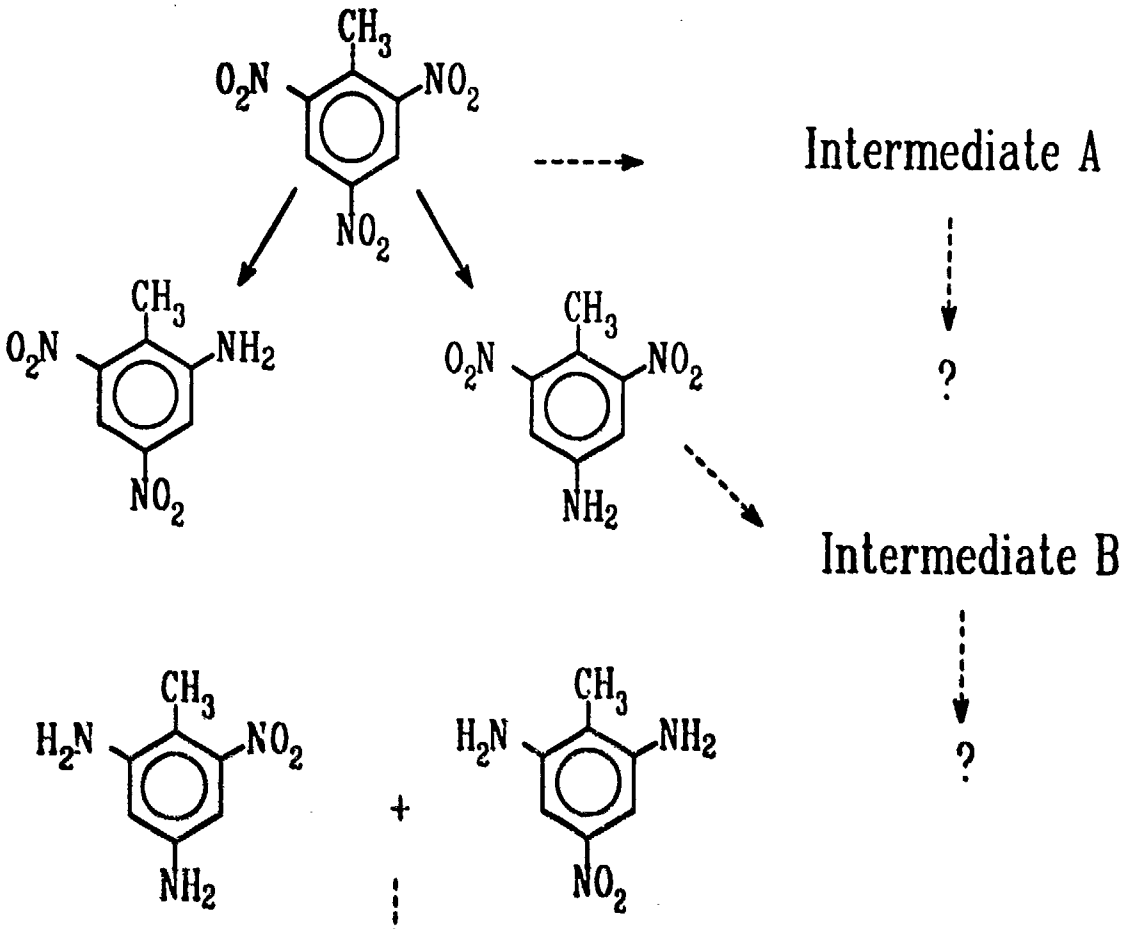


Figure 2

Time Course Analysis of Culture Supernatants

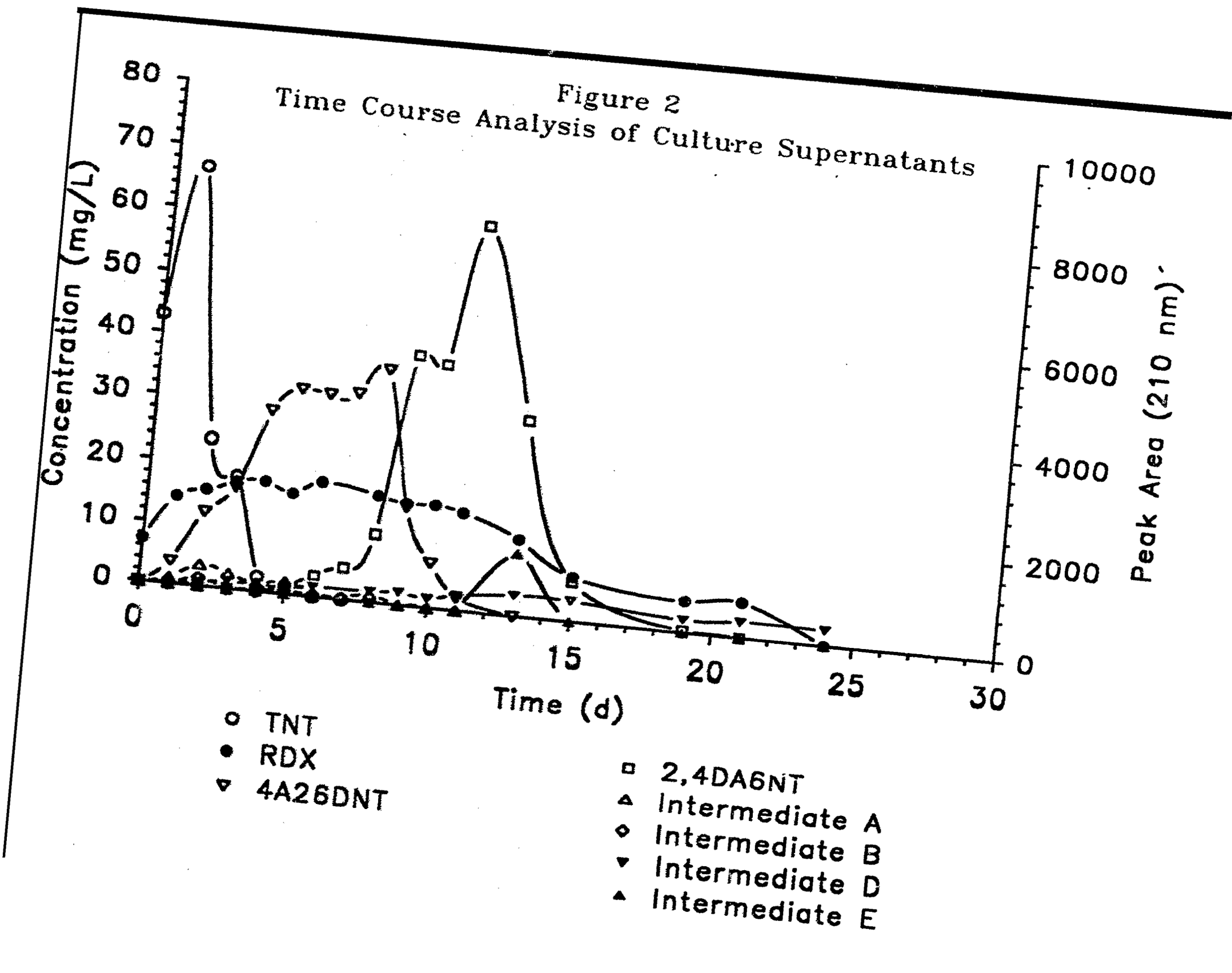


Table 1

Carbon Label Distribution in the Aqueous Phase (% of T=0 (sd))

Culture Condition	Retained on Filter	4A26DNT	2,4DA6NT	Total Fractions 1-3*	Total Recovery in Aqueous
Anaerobic pH 8	56.8 (2.2)	5.8	3.2	39.7	105.5
Anaerobic pH 6	nd	54	38.8	5.7	98.5
Aerobic	71 (10.6)	15.3	5.2	7.4	98.9

* All compounds not retained on the column and very polar intermediates are included in these fractions. e.g. VOA's and Intermediates D and E.

Physical Parameters Affecting the Anaerobic Degradation of TNT from Munitions-Contaminated Soil.

S.B. Funk, D.J. Roberts and R.A. Korus.

ABSTRACT

The bioremediation of 2,4,6-trinitrotoluene (TNT) and related explosive compounds in soils has become a promising line of research. Development of a biological remediation method for these soils would represent a major economic savings to the site owners. It has been estimated that physical treatment of munitions-contaminated soils in the United States alone will cost more than $1.5 billion. We obtained soil from a munitions site near Umatilla, Oregon. It was highly contaminated with TNT (12,000 mg/kg), RDX (3,000 mg/kg), and HMX (300 mg/kg). A procedure was developed in which the contaminated soil was mixed 1:1 w/v with phosphate buffer and a starch-rich carbon source. Indigenous aerobic organisms then utilized the starch and consumed oxygen, creating anaerobic conditions. This method has previously proven effective for the remediation of soils contaminated with dinoseb (2-*sec*-butyl-4,6-dinitrophenol). Initial experiments have shown that TNT also can be removed from soil using this procedure. We have determined the optimum ratio of soil to buffer to be 5 g soil/L buffer for use in bench-scale experiments, and examined the effects of manipulating physical parameters on the solubilization and degradation of TNT from contaminated soils. Temperature and pH strongly affected the solubilization of TNT from the soil as well as the biological activity within the soil slurries. Initial experiments reveal that the optimum temperature for biological activity is 35°C. The effects of addition of ammonium as a supplemental nitrogen source and varying starch/buffer ratios have also been studied.

INTRODUCTION

Culture conditons are very important to the growth and metabolic rate of microorganisms. Our goal is to optimize the rates of degradation while minimizing its cost. We are developing two-stage treatment methods using anaerobic to aerobic or nitrate reducing conditions.

There are many factors that affect the degradation rates of TNT and its metabolites. Culture parameters optimized include temperature, pH, buffer, nitrogen (as ammonium and nitrate), inoculum type and oxygen addition.

- aerobic methods have not proven complete remediation
- composting requires long incubation periods and creates large volumes of treated waste
- anaerobic slurries are the most promising method due to the increased contact between the microorganisms and the contaminants

METHODS

Culture conditions:

* 4 g of explosive-contaminated soil
* 400 ml of 50 mM phosphate buffer
* 4 g of potato processing by-product
* cultures were grown in 500 ml Erlenmeyer wide mouth flasks covered with aluminum and incubated in the dark

Analysis:

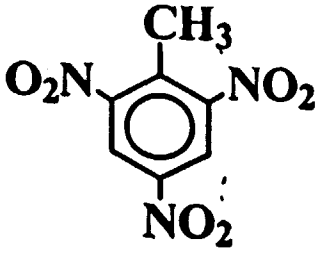
* aqueous phase of cultures were used for analysis
* HPLC using a C_{18} reverse phase column and equipped with a diode array detector
  - → 11 mM phosphate buffer (pH 4), acetonitrile solvent system
  - → 90% to 0% buffer in 17 min, 100% ACN 2 min, 0% to 90% buffer in 8 min
  - → detector set at 210 nm with continuous scanning of peaks from 190 to 600 nm
* pH was measured using an ion selective electrode
* ammonium and nitrate concentrations were measured using an ion selective electrode

DISCUSSION

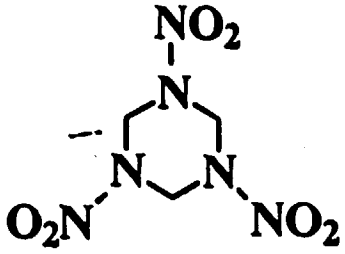
We have determined the optimal conditions for removal of TNT, RDX and their initial metabolic intermediates under anaerobic conditions. Implementation of these conditions has resulted in the bioremediation of munitions contaminants and their metabolic intermediates from soils within 24 days of incubation.

* rapid degradation was observed at temperatures between 20 and 35 °C and the optimal temperature was determined to be 30 °C
* ammonium chloride additions improved the removal of TNT, RDX and their metabolic intermediates over non-supplemented cultures, 25 mM was optimal
* rapid removal of TNT, RDX and their metabolic intermediates was observed at pH 8
  - → cultures grown at this pH formed large amounts of non-filterable material, presumably polymerized product
  - → this was not observed with cultures grown at pH 6 (Table 1)
* cultures incubated under aerobic conditions accumulated significantly higher amounts of the non-filterable material and had not removed all detectable intermediates by 24 days of incubation
* cultures incubated under anaerobic conditions then converted to aerobic or nitrate reducing conditions showed promising results
* future experiments will investigate new methods to reduce degradation time and prevent polymerization (pH 6)

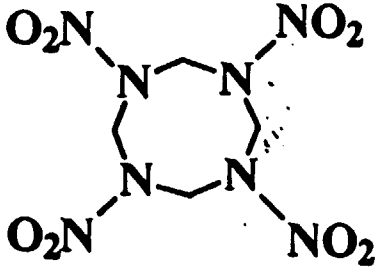
Structures



TNT



RDX



HMX

Table 1

Carbon Label Distribution in the Aqueous Phase (% of T=0 (sd))

Culture Condition	Retained on Filter	4A26DNT	2,4DA6NT	Fractions 1-3*	Total Recovery in Aqueous
Anaerobic phosphate buffer	56.8 (2.2)	5.8	3.2	39.7	105.5
Anaerobic nutrient broth	40.2 (3.9)	5.1	9.9	55.9	111.1
2-stage Anaerobic to nitrate	53.3 (3.2)	4.1	6.7	39	103.1
2-stage Anaerobic to aerobic	63.5 (6.8)	10.3	5.7	14.2	93.7
Aerobic	71 (10.6)	15.3	5.2	7.4	98.9
pH 6 anaerobic	nd	54	38.8	5.7	98.5

* All compounds not retained on the column and very polar intermediates are included in these fractions. e.g. VOA's and Intermediates D and E.

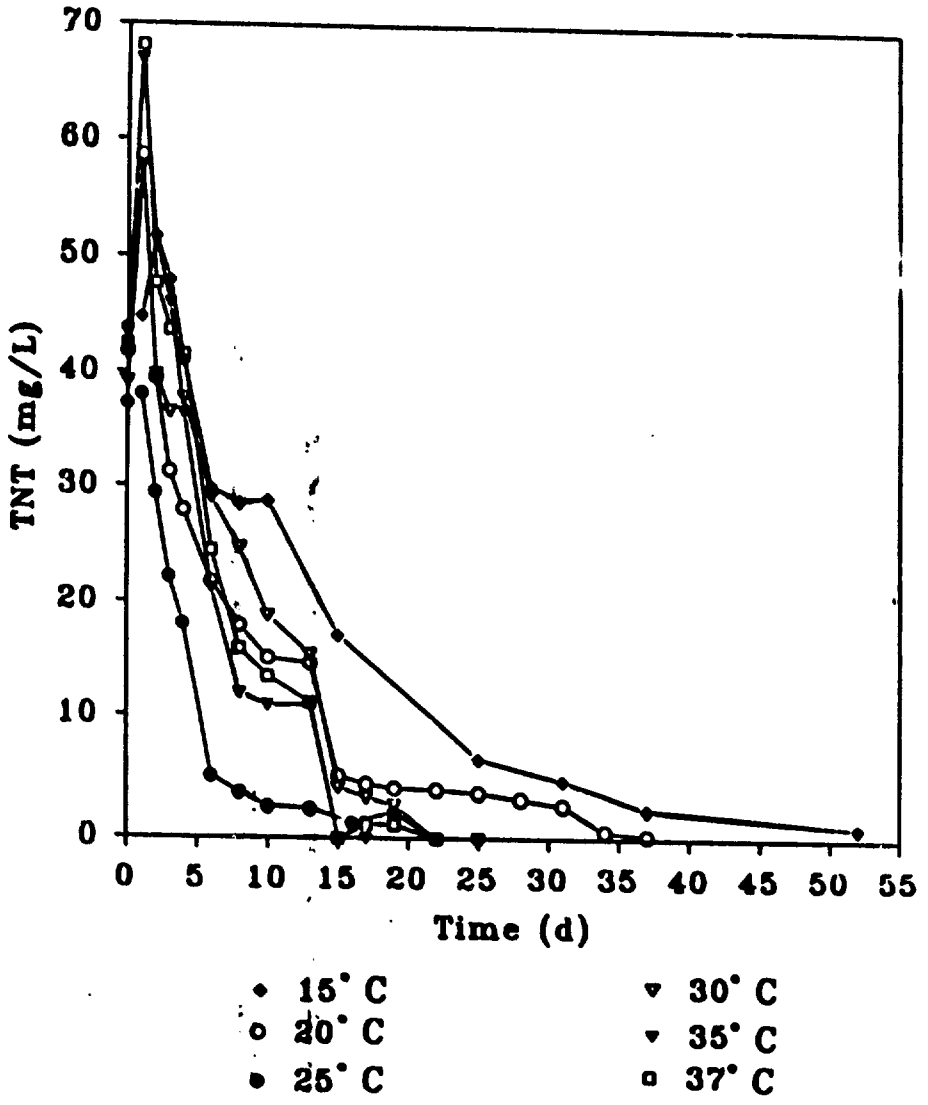


Figure 1.
Cultures were grown at temperatures between 15-45 °C. Cultures grown at temperatures between 25 and 35 °C exhibited a plateau showing little difference in the

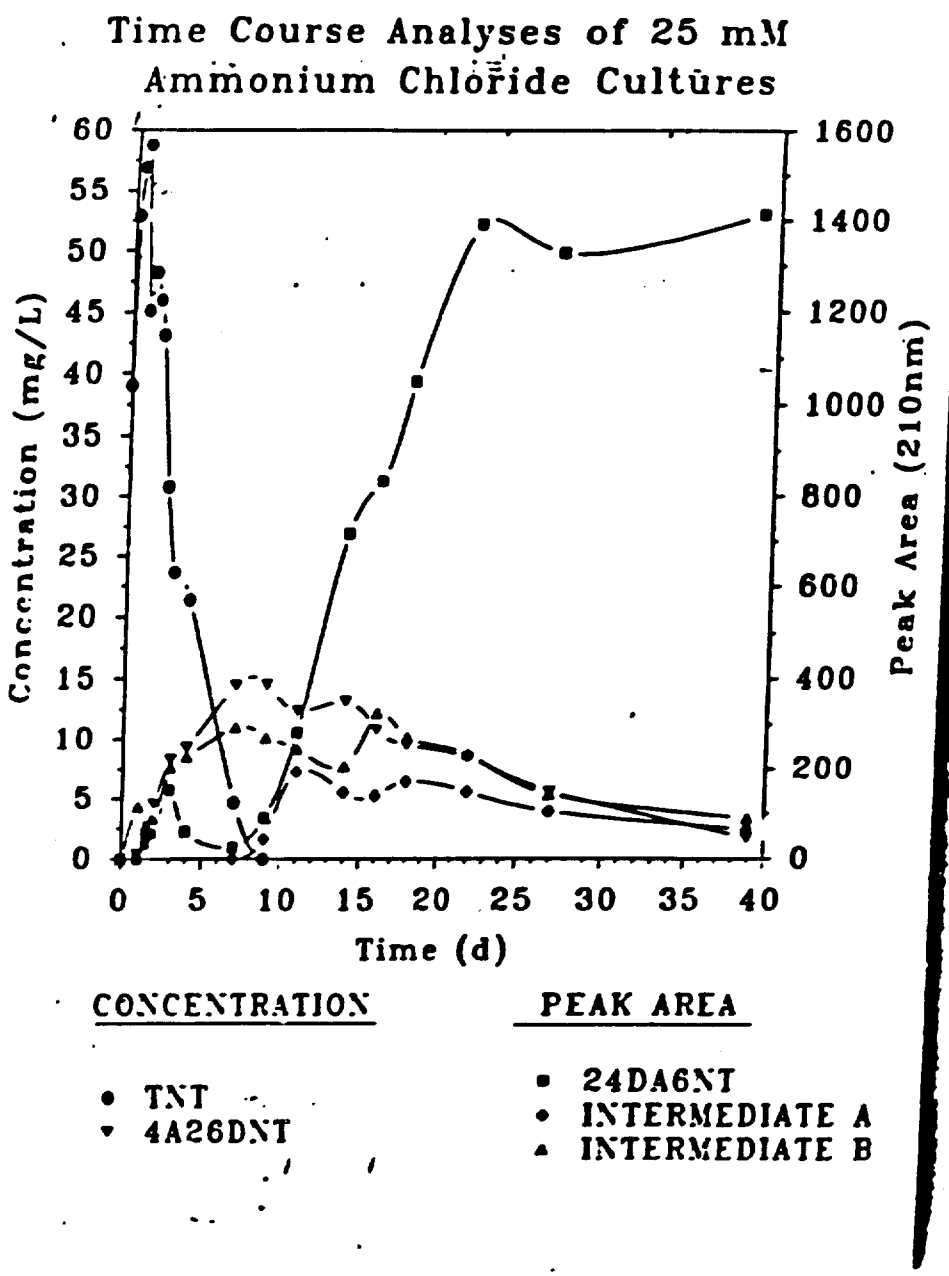


Figure 2.

Additions of ammonium chloride were made to cultures to see if an additional nitrogen source would increase the rate of degradation. Concentrations ranged from 0-30 mM and a concentration of 25 mM provided the best for degradation rates of parent compounds and the related intermediates

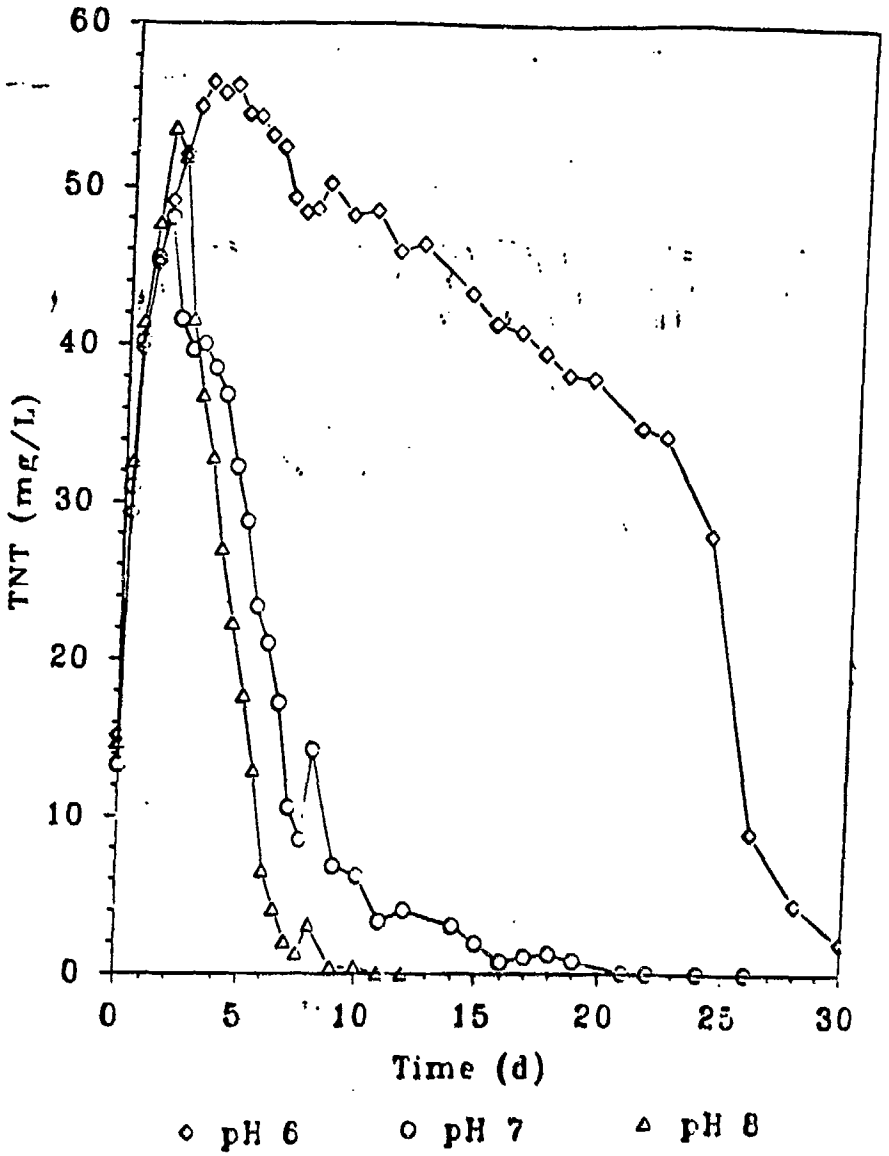


Figure 3.

Cultures were grown at pH 6, 7, and 8. Each culture's pH was measured and adjusted daily using phosphoric acid or potassium hydroxide. pH 8 was the optimal pH.

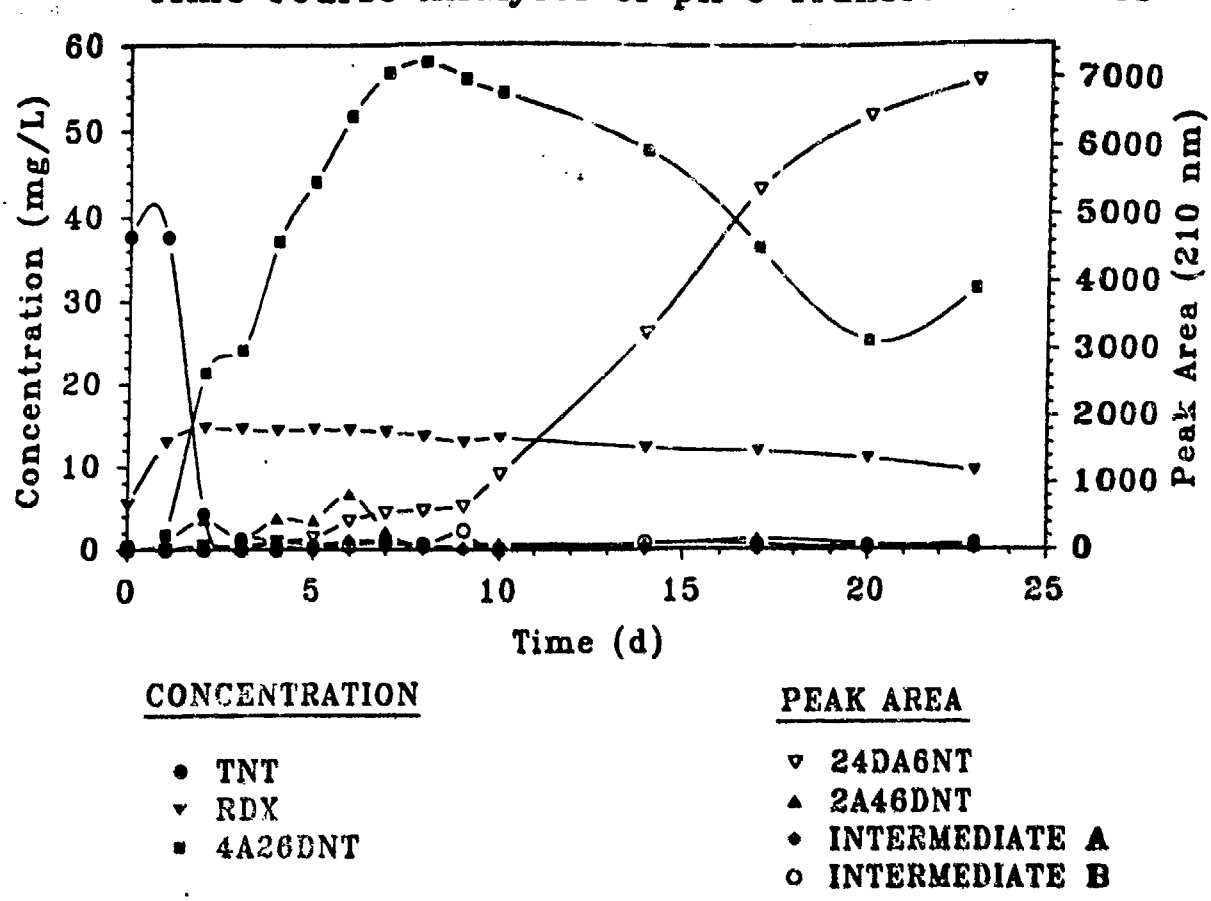


Figure 4.

Cultures were inoculated with 10 ml of aqueous solution from a culture grown at pH 6. Although pH 6 cultures showed slower degradation of TNT than pH 8 cultures, one of the replicates exhibited more rapid TNT removal than the others. This was used to inoculate these cultures.

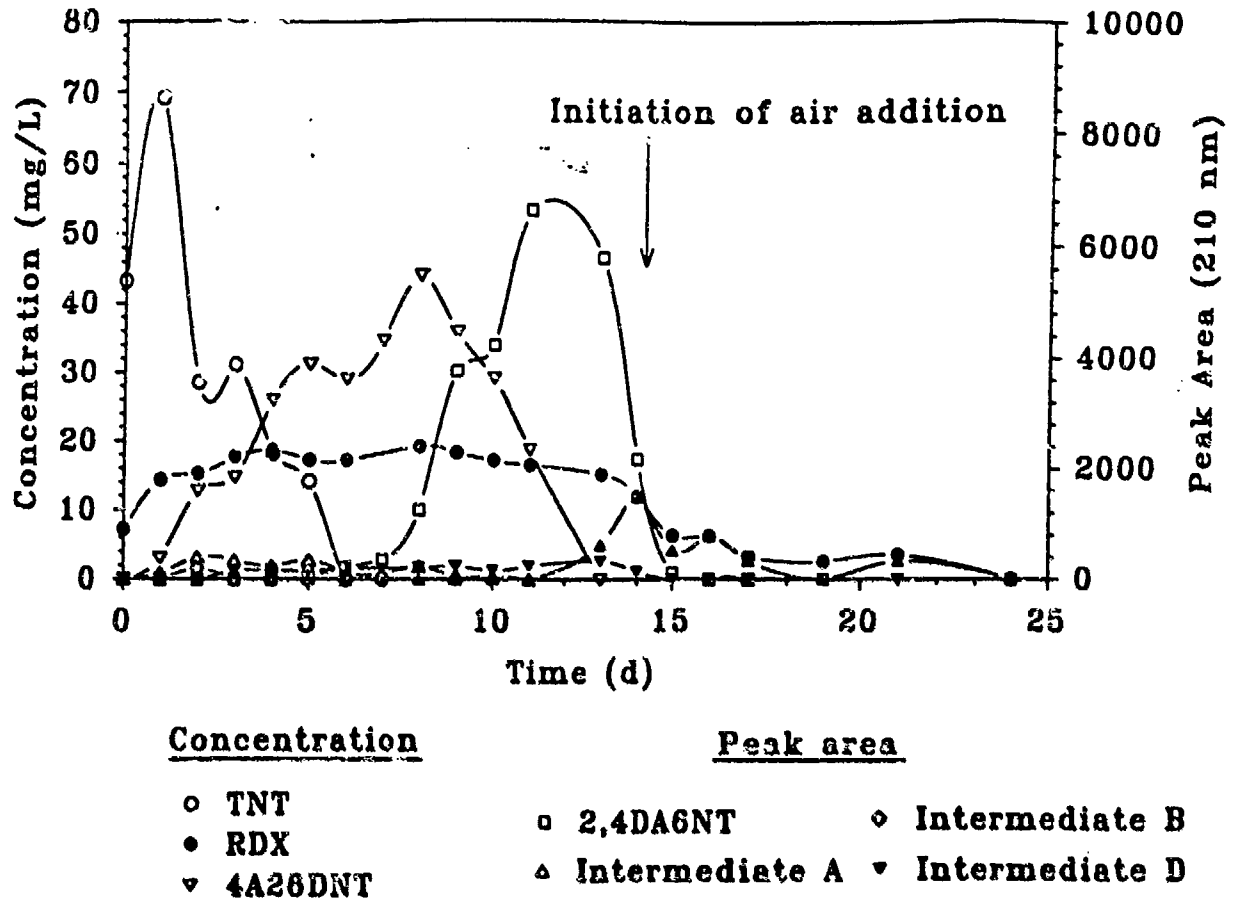


Figure 5.

The cultures were grown anaerobically for 14 days then conditions were made aerobic. The addition of 50 ml of air per day increased the removal of all monitored compounds within 24 days.

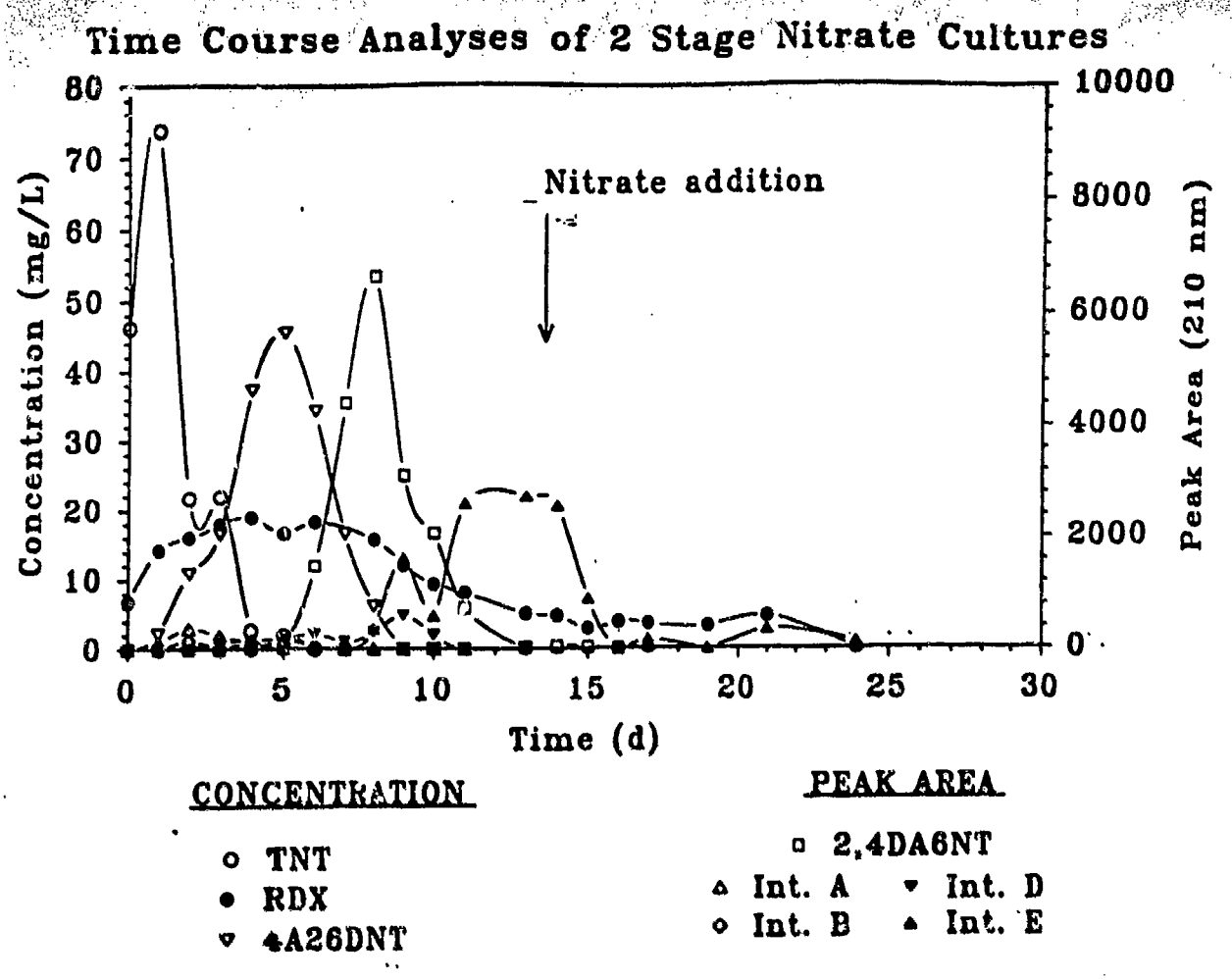


Figure 6.

Nitrate was added to a 5 mM final concentration at day 14 when the first intermediate, 4-amino-2,6-dinitrotoluene, was degraded. The results of this were similar to those of the air addition for removal of the remaining compounds.

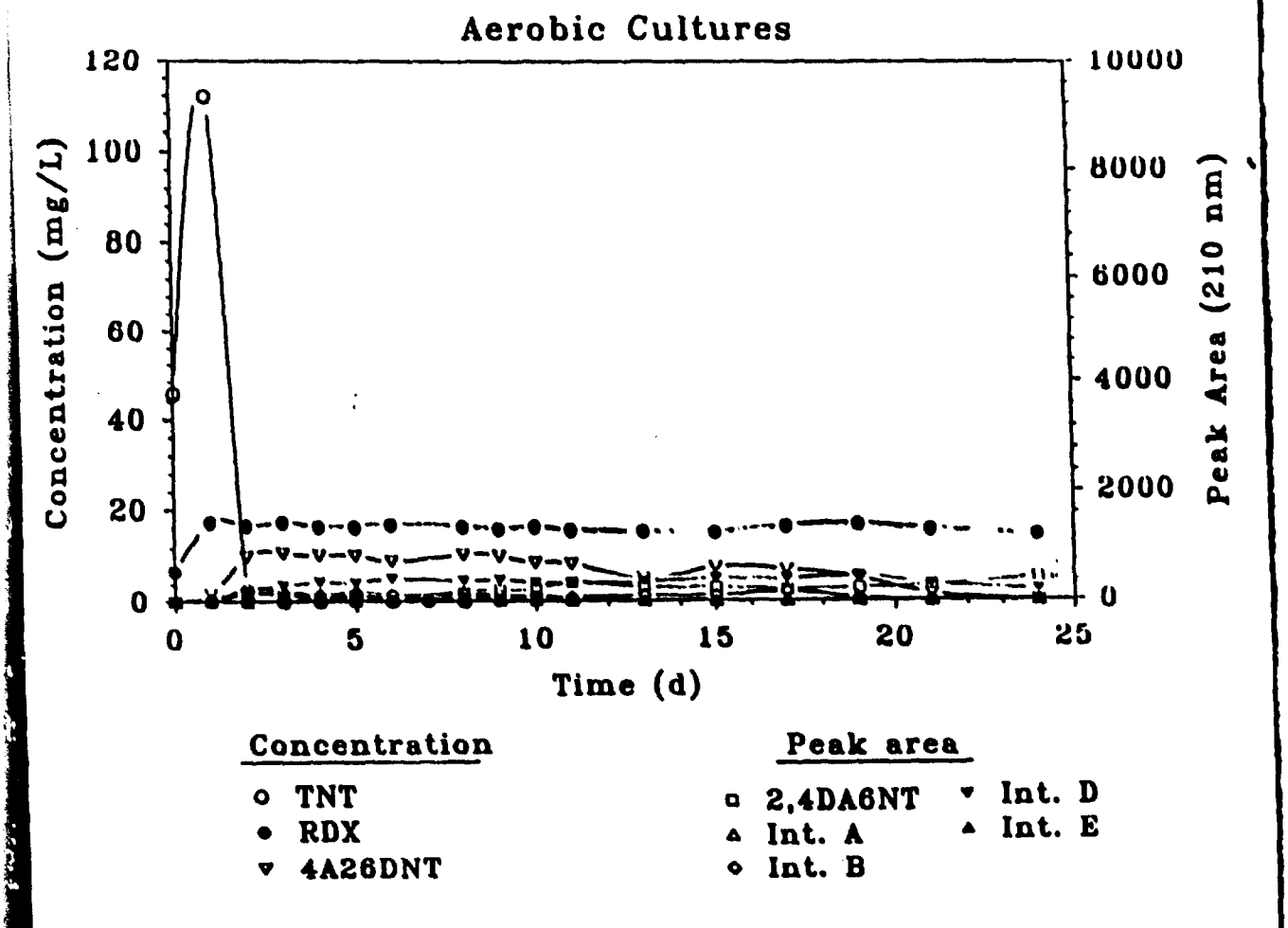


Figure 7.

Cultures grown aerobically showed rapid removal of TNT. Low levels of all intermediates formed but were not removed. These cultures have shown evidence of polymerization of the intermediates.

Intermediary Metabolism During Anaerobic Degradation of TNT from Munitions-Contaminated Soil.

D.J. Roberts, S.B. Funk and R.A. Korus.

ABSTRACT

The anaerobic degradation of TNT and other nitroaromatic compounds in contaminated soils represents an inexpensive alternative to conventional remediation technology for these soils. Metabolism of TNT under aerobic conditions promotes the formation of polymerization products from unstable hydroxylamino intermediates formed during non-specific reductions of the nitro groups. Anaerobic conditions circumvent this polymerization because reductions proceed rapidly and completely to the amino compounds. Most studies concerning the metabolism of nitroaromatic compounds having multi-nitro substituents have used low concentrations of pure compounds and have shown that metabolism of these compounds usually stops after formation of the amino compounds. Munitions-contaminated soils usually contain multiple compounds, often in very high concentrations. We studied th bioremediation of a soil contaminated with 15,000 mg total explosives/kg soil. Contaminants included the compounds TNT, RDX and HMX. We demonstrated the removal of TNT and the early reductive intermediates formed during anaerobic TNT metabolism. ^{14}C-U-ring-labeled TNT was used to study the mineralization and production of intermediates from TNT during incubations of TNT-contaminated soil under anaerobic conditions. Intermediates were isolated by solvent extraction and chromatography techniques and were identified by mass spectroscopy. Important intermediates included 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, and 2,6-diamino-4-nitrotoluene, and at least two other very polar intermediates.

INTRODUCTION

- Munitions contaminated soils are a very large contamination problem in the U.S.
- Bioremediation of contaminated soils is a very attractive alternative to physical remediation methods.
- Our anaerobic process has been applied successfully to the bioremediation of soils contaminated with another nitroaromatic compound dinoseb (2-*sec*-butyl-4,6-dinitrophenol).
- Understanding the intermediary metabolism of the contaminants is a very important part of the design of an effective treatment procedure.
- The majority of the research to date has indicated that the transformation of TNT is through simple reduction reactions which only convert TNT to amino-nitro derivatives.
- Development of a system capable of carrying out more complete or different types of transformations of TNT will lead to a better bioremediation process.

METHODS

Culture Conditions

* Soil experiments
  - → 1% soil in mineral medium or phosphate buffer.
  - → inoculated with dinoseb acclimated soil or acclimated aqueous culture.
* Acclimated Aqueous Culture
  - → a 4 L anaerobic culture obtained from nitroaromatic contaminated soil and anaerobic sewage sludge.
  - → maintained for 2 years on a feed solution of munitions compounds extracted from contaminated soil.
* Labeled experiments
  - → C^{14}-[U-ring]-labeled TNT purified to 98% by TLC.
  - → C^{14} as intermediates separated by HPLC, 1 min fractions collected and C^{14} determined by LSC.
* Extraction of Intermediates
  - → Aqueous phases of cultures at various stages of degradation exhaustively extracted with ethyl acetate.
  - → Organic phases analyzed by GC/MS directly.
  - → Aqueous phases air dried, dissolved in methanol, analyzed by GC/MS directly or as silyl-derivatives or by HPLC/MS directly.

Analysis

* HPLC Analyses
  - → C_{18} reverse phase column
  - → 11 mM phosphate buffer (pH 4), acetonitrile solvent system.
  - → 10% acetonitrile for 2 min, 10% to 85% acetonitrile over 15 min, 85% to 100% acetonitrile over 2 min, 100% acetonitrile maintained for 2 min, 100% to 10% acetonitrile over 2 min.
  - → Detection via diode array detector at 210 nm.
  - → Spectra from 200-600 nm saved for each peak.
* MS Analyses
  - → GC - HP-1 12m fused silica capillary column, temperature programmed from 120 °C (1 min) to 300 °C (2 min) at 10 °C per minute.
  - → LC - C_{18} reverse phase column, 100% water for 3 min, 100% water to 100% acetonitrile over 21 min.
  - → MS - HP 5898A MS, Electron Impact Ionization with electron energy at 70 v and repellor at 7 v. The MS ion source was set at 200 °C and the Quadrapole detector temperature at 100 °C. Data collected with photomultiplier volts at 400 above calibration and from 50 - 400 mz.

Description of Intermediates

* *Intermediate A*

Accumulates to a very small extent during the early stages of TNT degradation. UV/Vis spectrum indicates nitro-groups still present on an the aromatic ring. HPLC retention time of 14.9 min indicates that the compound is less polar than TNT. Has not accumulated in sufficient quantities to obtain a mass spectrum.

* *Intermediate B*

Accumulates during the early stage of TNT degradation. UV/Vis spectrum indicates nitro- and possibly amino-groups present on the aromatic ring. HPLC retention time of 8.4 min indicates that the compound is more polar than TNT. Has not accumulated in sufficient quantities to obtain a mass spectrum.

* *2,4-diamino-6-nitrotoluene (Intermediate C)*

Has been identified by GC/MS of ethylacetate extract of culture supernatants. Although no authentic standard for this compound has been obtained the identity was derived by comparison of UV/Vis and mass spectra and HPLC and GC retention times to those of an authentic standard of 2,6-diamino-4-nitrotoluene. The UV/Vis and mass spectra were similar enough to confirm the functional groups were the same, the differences in spectra and retention times indicated the two were different isomers.

* *Intermediate D*

Accumulates during the later stages of TNT degradation. UV/Vis spectrum indicates that amino-, hydroxy-, and nitro-groups are possibly present on the aromatic ring. The compound is very polar (HPLC retention 2.5 min). It has been purified to a large extent by extraction techniques. It is not soluble in ethyl acetete or acetonitrile to any extent but is soluble in water or methanol. A mass spectrum was not obtained from samples analyzed by GC/MS with and without silanization nor from HPLC/MS even though a large peak was observed with the UV detector of the HPLC.

* *Intermediate E*

Accumulates during the later stages of TNT degradation. UV/Vis spectrum indicates that this compound probably does not contain any nitro groups. The influence of the amino groups on the spectrum is also greatly reduced. The compound is very polar (HPLC retention time 1.8 min). A mass spectrum was not obtained from samples analyzed by GC/MS with and without silanization nor from HPLC/MS even though a large peak was observed with the UV detector of the HPLC.

* *Intermediate F*

Accumulates during the later stages of TNT degradation. UV/Vis spectrum indicates the compound is phenolic in nature. Possibly a dihydroxytoluene isomer. The compound is less polar than intermediate E.

* *p-Cresol*

Accumulates in the very late stages of the degradation of TNT. Has been identified by comparison of UV/Vis spectrum and HPLC retention time. Accumulates frequently in the large TNT adapted aqueous inoculum.

DISCUSSION

* We have achieved degradation of TNT to intermediates that, although are still aromatic in nature, are much more amenable to degradation under both anaerobic and aerobic conditions.
* The degradation of TNT by our anaerobic cultures appears to proceed through at least two pathways or a network of intermediates.
* The major route of degradation appears to be through successive reduction of the *para*- then an *ortho*-nitro group to the amino derivatives. The diamino compounds are themselves degraded producing highly polar compounds. 2-methyl-1,3,5-trihydroxybenzene has been identified previously in the culture supernatants of early enrichment cultures but does not accumulate in more adapted cultures. This compound is apparently dehydroxylated to *p*-cresol which accumulates to some extent in the culture supernatants.
* The continued accumulation of *p*-cresol in the culture supernatants of well adapted cultures indicates that the organisms that degrade this compound are inhibited by some compound in the metabolic pathway of TNT degradation. The acclimated aqueous culture accumulates *p*-cresol for a short period of time and then, apparently, the inhibition is overcome and then a rapid removal of *p*-cresol is observed, accompanied by gas production. We assume that given time the population will develop to a point where the members of the consortium are completely adapted to the various compounds formed during TNT degradation and then the metabolism will proceed rapidly without accumulation of intermediates.
* The importance of the minor intermediates A and B have not been determined. They may represent an alternative pathway which may prove to be important. Since this pathway represents an alternative to reduction reactions which produce intermediates that are unstable and polymerize to form more recalcitrant compounds it may prove of importance.

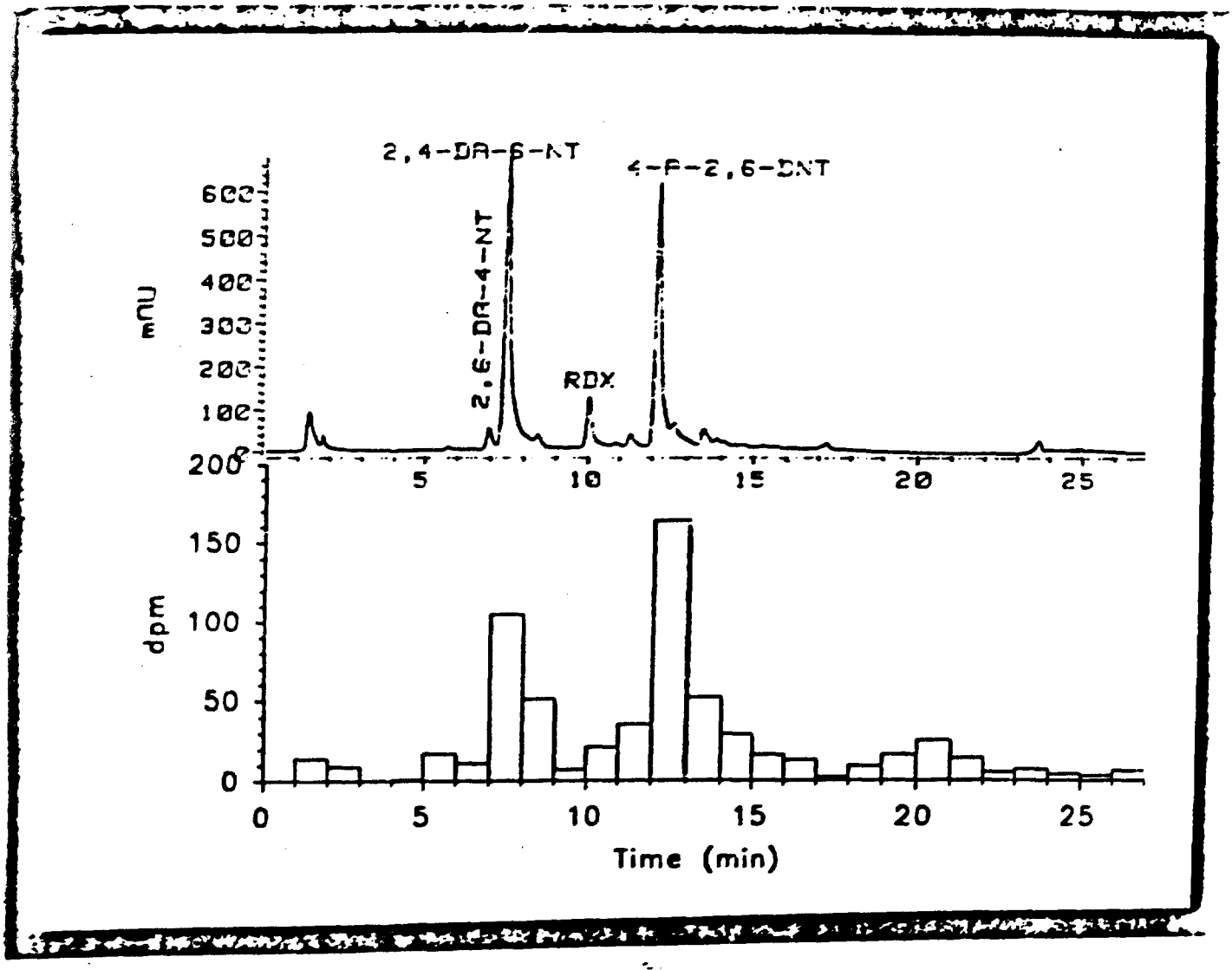


Figure 1.

Recovery of C^{14} from C^{14}-[U-ring]-labeled TNT as intermediates. Early in the degradation period. UV chromatograph recorded at 210 nm. 1 min fractions were collected and analyzed by LSC.

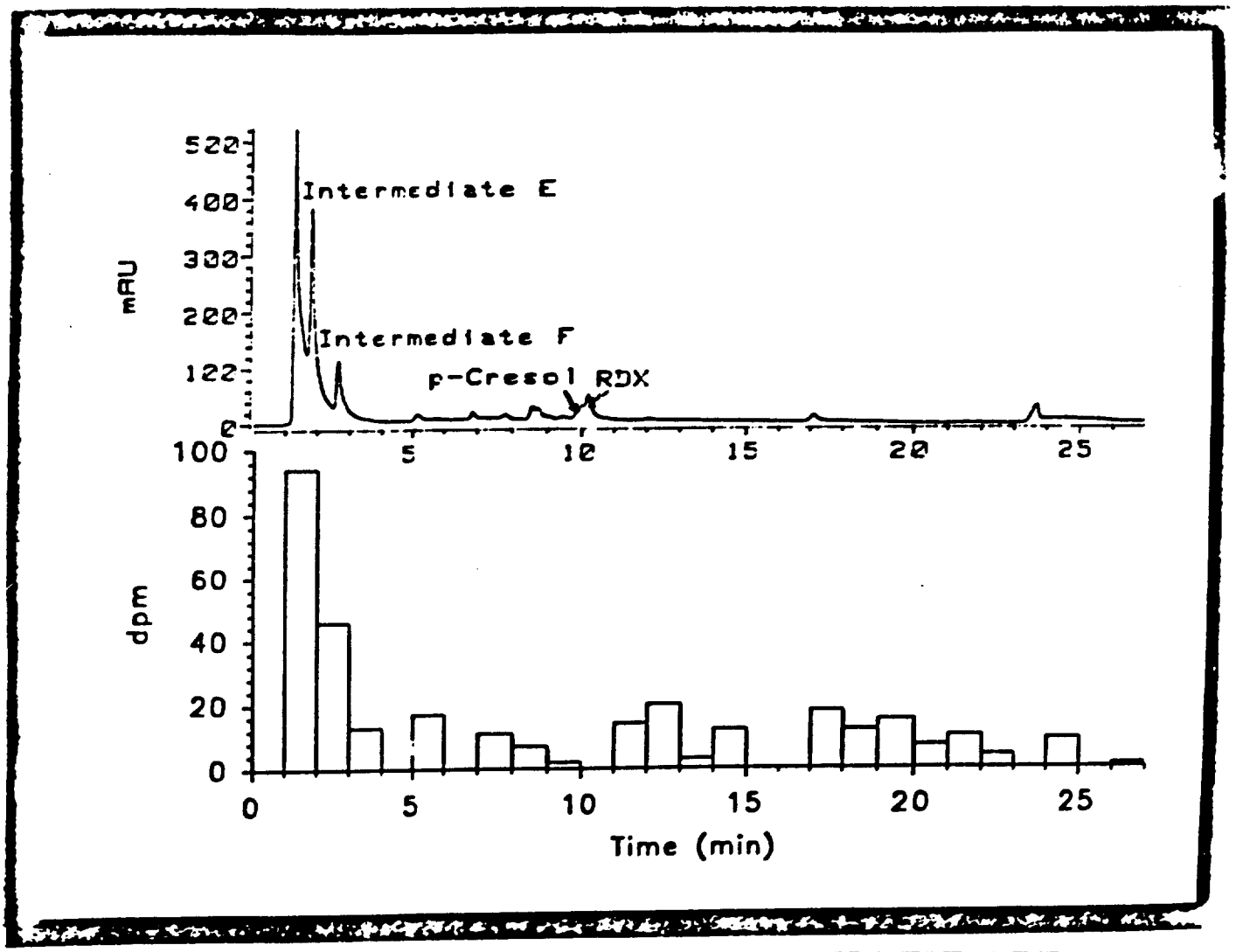
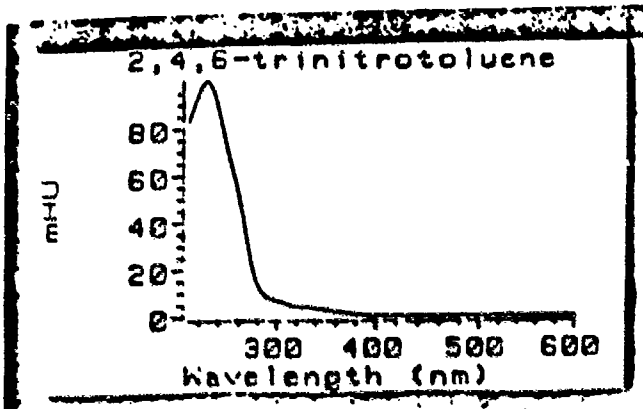
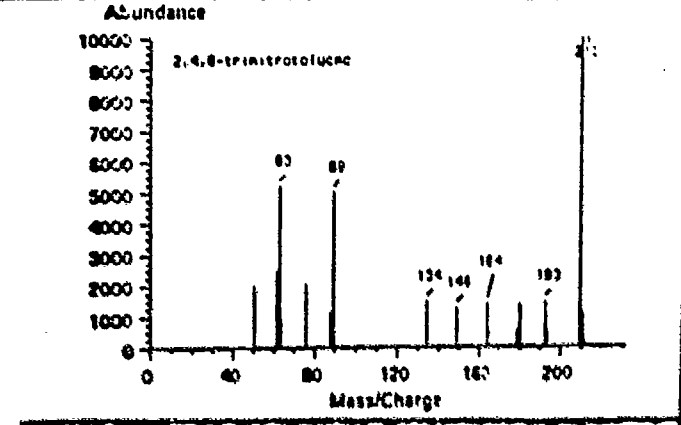
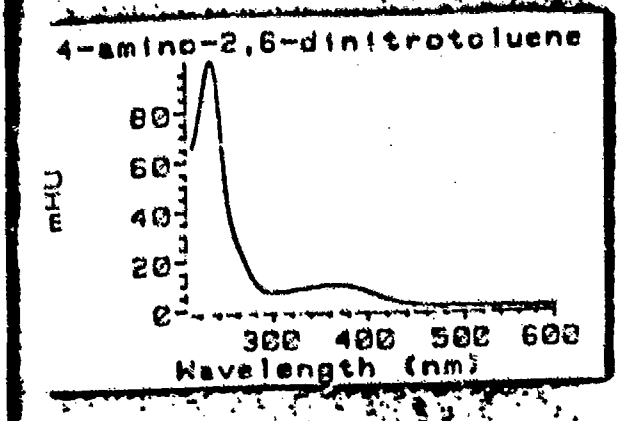


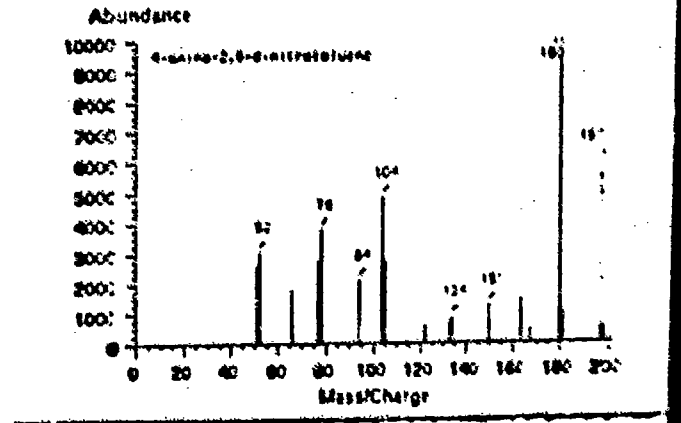
Figure 2.

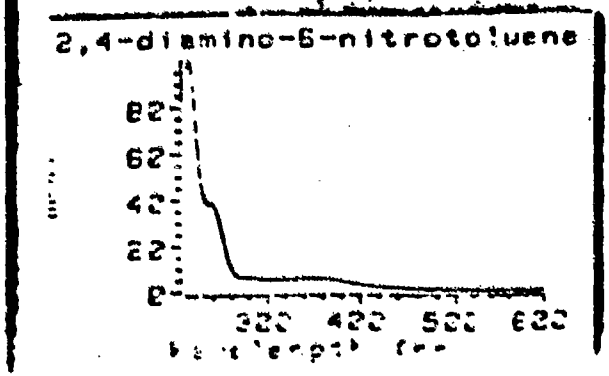
Recovery of C^{14} from C^{14}-[U-ring]-labeled TNT as intermediates. Late in the degradation period. UV chromatograph recorded at 210 nm. 1 min. fractions were collected and analyzed by LSC.

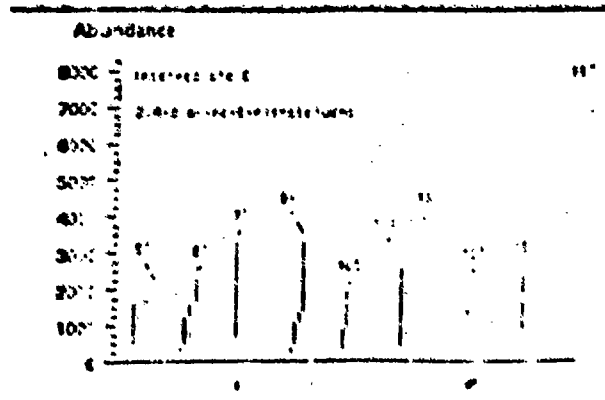

2,4,6-trinitrotoluene
mAU
80
60
40
20
0
300 400 500 600
Wavelength (nm)


Abundance
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0 40 80 120 160 200
Mass/Charge


4-amino-2,6-dinitrotoluene
mAU
80
60
40
20
0
300 400 500 600
Wavelength (nm)


Abundance
0 20 40 60 80 100 120 140 160 180
Mass/Charge


2,4-diamino-5-nitrotoluene
82
62
42
22
2


Abundance

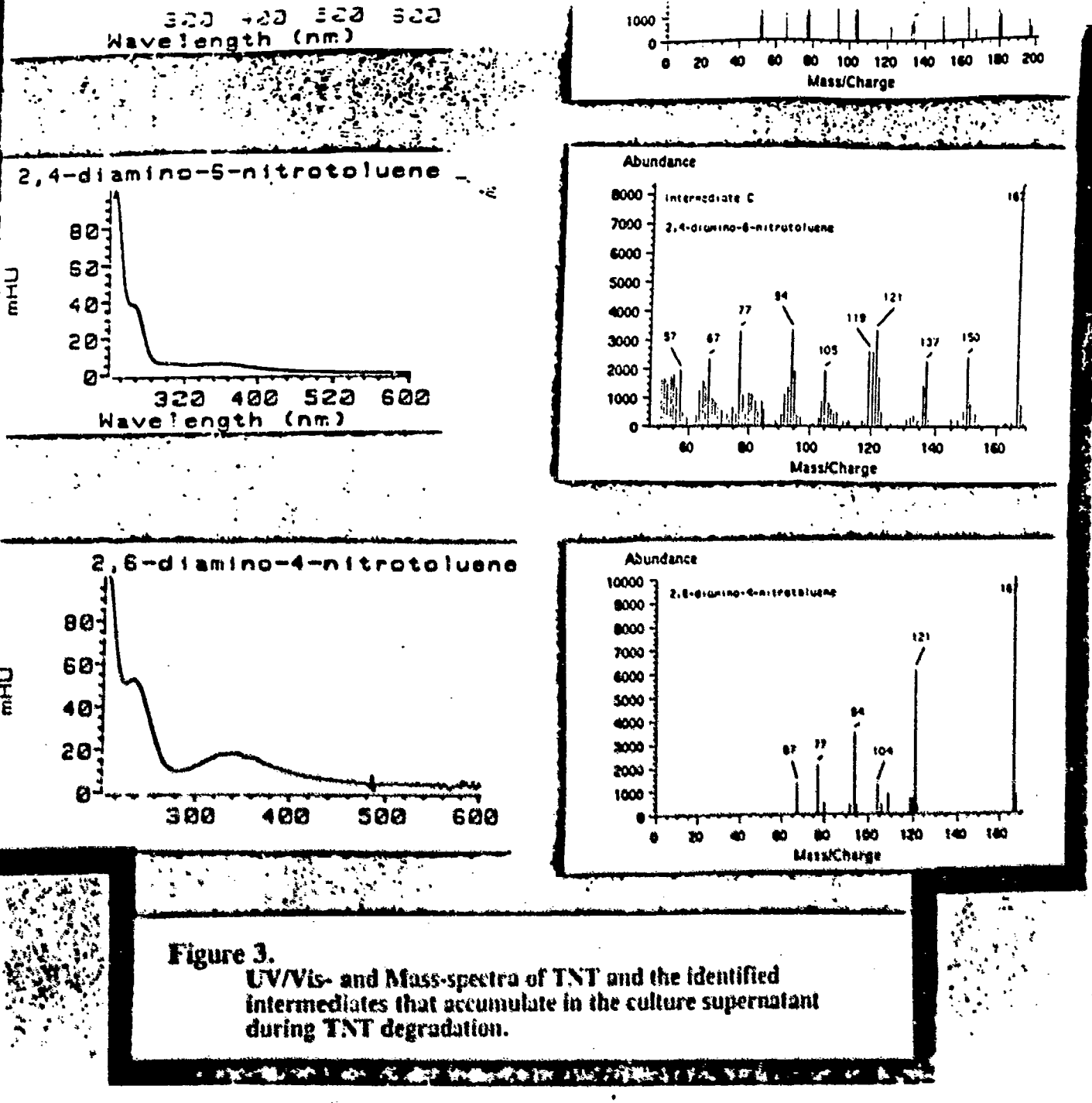


Figure 3.
UV/Vis- and Mass-spectra of TNT and the identified intermediates that accumulate in the culture supernatant during TNT degradation.

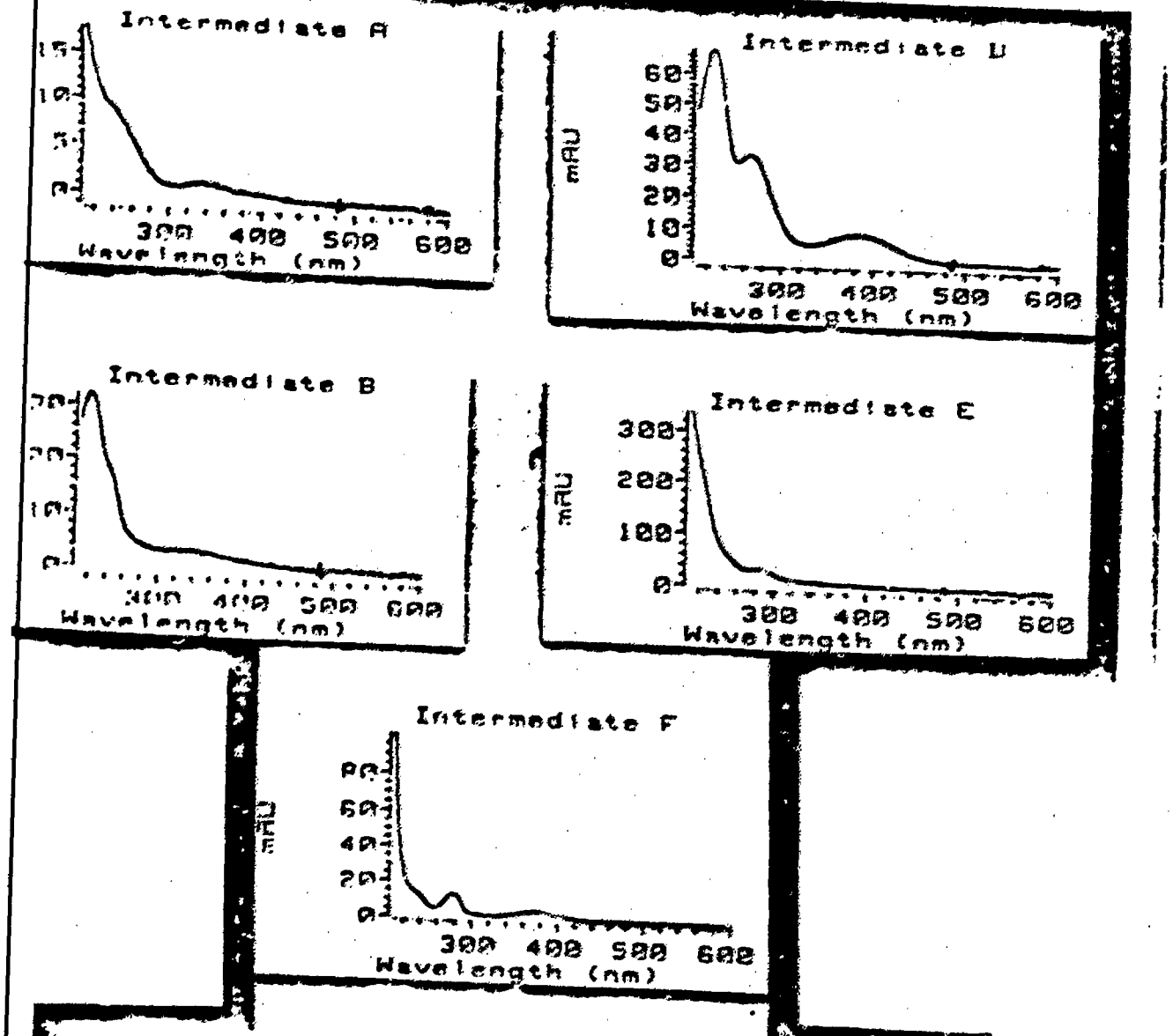


Figure 4.
UV/Vis spectra of the major unidentified compounds accumulating in the culture supernatant during TNT degradation.

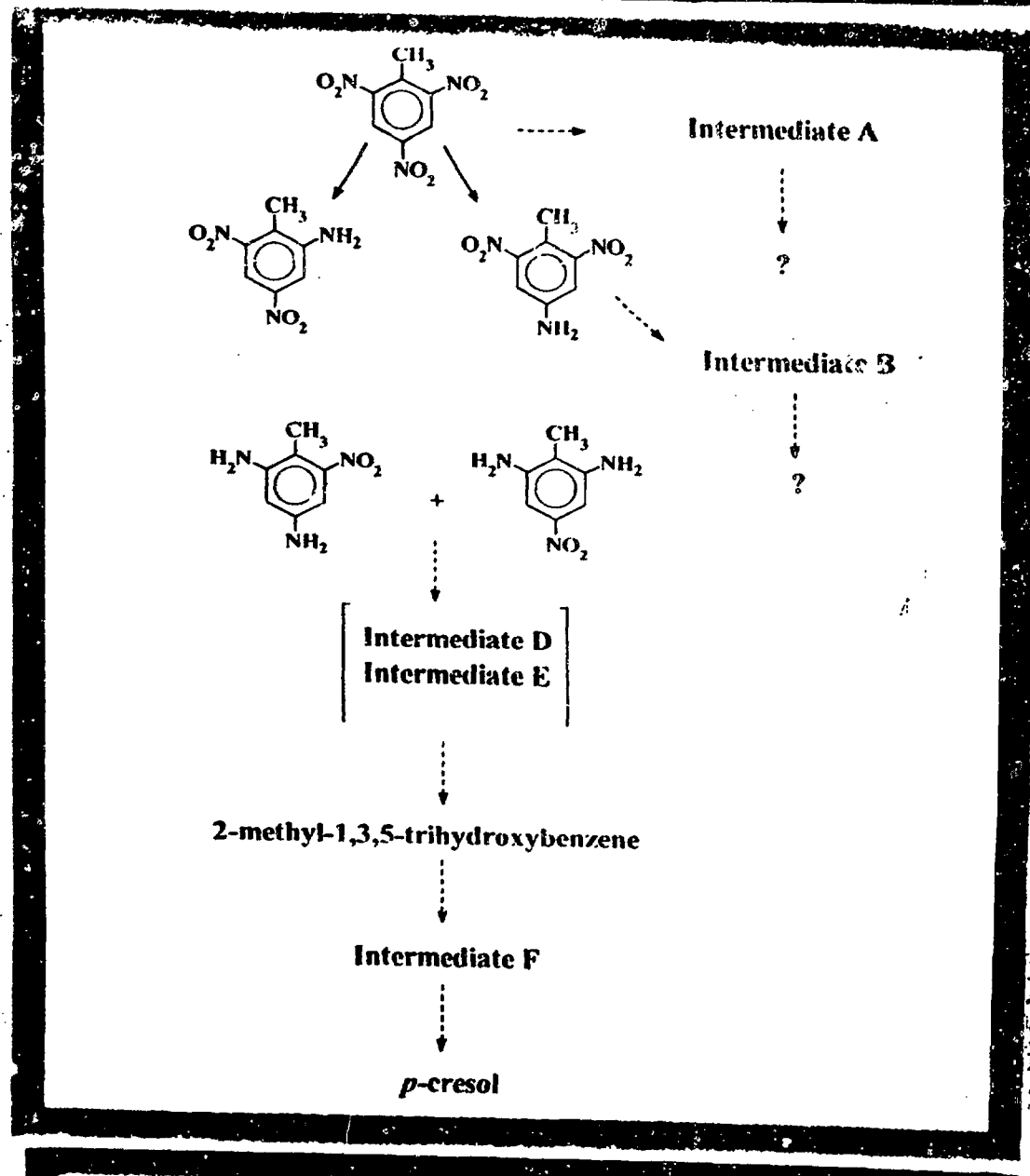


Figure 5.
Possible network relating compounds accumulating in culture supernatant during TNT degradation. Dashed arrows indicate speculative relationships.